12/PRTS

A NOVEL LYMPHOCYTE POPULATION EXPRESSING INTRACELLULAR MO2

INS A1

FIELD OF THE INVENTION

The invention concerns a novel population of lymphocytes and further concerns use of said lymphocytes for various diagnostic assays.

PRIOR ART

The following is a list of prior art publications referred to in the present specification.

1. Simmons, David, L., *et al., Blood*, __73__:284-289, 1988.

2. Goyert, S.M., *et al., Science*, __239__:497-400, 1988.

3. Haziot A., *et al., J. Immunol.*, __141__:547-552, 1988.

4. Lauener, R.P., WO Patent Application No. 93632418.

5. Hans, *et al.*, WO Patent Application No. 9525117.

6. Dietzh, *et al.*, German Patent Application No. DE 4237502.

7. Landsorf, P. and Thomas, T., Canadian Patent Application No. 2191655.

8. Calder, V.L. *et al.*, *5th International Conference on Human Leukocyte Differentiation Antigens, Boston, Mass., U.S.A.*, 1993

9. Bernard, A., *et al., Leukocyte Typing: Berlin*, __108__:425-426, 428, 1984.

10. McMichael A.J., *Leukocyte Typing III, White Cell Differentiation Antigens*, 586-589, 1987.

11. Todd, R.F., *et al., Hybridoma*, **1**:329-337, 1982.

12. Todd, *et al., J. Immunol.*, **126**:1435-1442, 1981.

13. Todd, R.F., and Schlossman, S.F., *Blood*, **59**:775-786, 1982.

14. Todd, *et al., Leukemia Res.*, **5**:491-495, 1981.

The acknowledgement herein of the above art should not be construed as an indication that this art is in any way relevant to the patentability of the invention as defined in the appended claims.

The above publications will be acknowledged in the following by indicating their number from the above list.

BACKGROUND OF THE INVENTION

Identification of specific cell types and populations is necessary and desired for correct diagnosis of various disorders and diseases and enables more correct and efficient prognosis and treatment of such conditions.

Many specific populations and sub-populations of lymphocytes are known today which are identified typically by use of monoclonal antibodies that are capable of specifically recognizing antigens expressed by such cells.

CD14 is a glycosyl-phosphatidylinositol-linked single-chain surface membrane glycoprotein with a molecular weight of about 55 kDa(1). It is expressed to a high extent on monocytes, macrophages and to a low extent on neutrophils. It has been shown to be very weakly expressed on the outer membrane of certain B-cell lymphocytes, but is absent from T-cells, null cells, red blood cells and platelets. CD14 was also described to be expressed on the membrane of Langerhans cells, follicular dendritic cells and histiocytes, and to be present in a soluble form in serum. CD14 has been

shown to be a receptor for lipopolysachharide (LPS) and is considered to be a myelomonocytic differentiation antigen[2,3].

Anti CD14 MAbs have been shown to induce IL-10 secretion from CD14 expressing cells or by inducing T-cell tolerance or anergy[4]. Anti-CD14 antibodies were also described for use in the prevention or treatment of various bacterial infections such as toxemia associated with gram-positive bacteria infection, especially sepsis[5]. Medicaments containing anti-idiotype CD14 antibodies having high endotoxin binding capacity were described for the treatment of infection and other disorders[6].

Compositions comprising a combination of a number of antibodies including anti-CD14 antibodies have also been described for use in the production of enriched human blood cell preparations as well as for the enrichment of non-hematopoietic metastatic tumor cells[7].

A number of CD14 epitopes have been reported, each being recognized by a specific MAb. Such Mabs are, for example, My4[8, 10], CMRF-31[8] and MO2 [9-14] (hereinafter *"the MO2 MAbs"*). Intracytoplasmic CD14 protein recognized by the My4 and CMRF-31 antibodies was reported in some non myeloid cells[8].

The MO2 MAb has been shown to react with 58%-84% of peripheral blood monocytes. The MO2 antigen was shown to be expressed on 6%-16% of bone marrow cells, on mature monocytes and on certain macrophages, but is not known to be expressed by T or B lymphocytes or by granulocytes.

SUMMARY OF THE INVENTION

In accordance with the present invention, a novel population of lymphocytes was surprisingly discovered comprising cells that express intracellularly, an antigen recognized by the MO2 Mab. Such cells will be referred to hereinafter as *"MO2$^+$ cells"*, and the antigen expressed intracellularly by such cells will be referred to as *"MO2 antigen"*.

The novel population of cells was detected using the known MO2 antibody and the expression of this antigen in these cells was detected following their fixation and permeabilization. No expression of the MO2 antigen was detected on the cell surface of these cells. Therefore, unless specifically stated otherwise, reference to the expression of the MO2 antigen by cells will refer to intracellular expression of the antigen.

The novel cell population of the invention was found to comprise two subpopulations. One subpopulation of cells, in addition to expressing the MO2 antigen internally, was also found to express the general T-cell antigen marker CD3 and therefore this population of cells is considered to be a T-cell population of lymphocytes and will be referred to hereinafter as $CD3^+$ $MO2^+$ cells. In accordance with the invention, it was also found that a large percent of T-cells in peripheral blood which express the CD8 antigen (characterizing mainly suppressor and cytotoxic T-cells) also express the MO2 antigen intracellularly. Only a small percent of peripheral blood T-cells expressing the CD4 antigen (characterizing T-helper cells) were found to express the MO2 antigen intracellularly.

T-cells expressing the gamma/delta T-cell receptor (TCR) are believed to be involved in the etiology of infectious diseases. In accordance with the invention, it has been shown that most of these T-cells expressing the gamma/delta TCR also express the MO2 antigen intracellularly.

The second novel subpopulation of lymphocytes which, in accordance with the invention were found to express the MO2 antigen internally, are characterized as non-T, non-B cells. At least some of these cells express the CD16 antigen marker (and thus may be NK or NK-like cells).

The present invention thus provides a novel population of lymphocytes which express intracellularly a protein or polypeptide which binds to an antibody being a member of the group consisting of:

(i) anti MO2 MAbs;

(ii) fragments of the antibodies of (i) above which essentially retain the antigen binding characteristics of the non fragmented anti MO2 MAb;

(iii) antibodies which bind to the antigenic epitope bound by any one of the antibodies of (i) or (ii) above;

said lymphocytes having no expression of the MO2 antigen on their surface.

By one embodiment, the novel population of $MO2^+$ cells in accordance with the invention are T-cells expressing the CD3 antigen on their cell surface and expressing the MO2 antigen intracellularly, hereinafter $CD3^+$ $MO2^+$. The expression of the CD3 antigen on these cells can be detected by any of the methods known in the art using any of the anti-CD3 MoAbs available such as, for example, the MoAb manufactured by Coulter Immunology. The $MO2^+$ T-cells comprise additional subpopulations which express, in addition to the MO2 antigen, also CD4 (helper cells), CD8 (suppressor cells) or gamma/delta antigen.

The present invention further provides a novel population of non-T non-B $MO2^+$ lymphocytes. Such cells may be detected by their positive reaction after fixation and permeabilization with the anti MO2 MAb, and by their negative reaction with anti-CD3 Mabs (a general marker of T-cells) as well as their negative reaction with anti-CD22 MAbs (a general marker of B cells) Some of these $CD3^-MO2^+$ cells were found to react with anti CD16 MoAb (a marker of natural killer (NK) and NK-like cells.)

Moreover, in accordance with the invention, a significant increase was shown in the percent of $MO2^+$ T-cells and or in the level of MO2 expression in peripheral blood cells of individuals suffering from infections of viral or bacterial origin as compared to the percent of such cells and/or level of intracellular MO2 in the same cells in peripheral blood of healthy individuals. Further analysis showed that in many cases the most significant increase was

in the $MO2^+$ $CD3^+$ cells (including both $CD4^+$ $MO2^+$ cells and $CD8^+$ $MO2^+$ cells) of the infected individuals. .

The present invention further provides a method for detection of an individual with a high probability of having an infection comprising the steps of:

(i) obtaining a peripheral blood sample from said individual;

(ii) separation of mononuclear cells from said peripheral blood;

(iii) fixation and permeabilization of said mononuclear cells;

(iv) incubation of said fixed and permeabilized mononuclear cells with MAbs which bind to the MO2 antigen under conditions enabling binding of the Mabs to said antigen;

(v) detecting binding of said antibodies in said cells, and determining the number of cells in said sample expressing the MO2 antigen internally and/or the intracellular level of MO2 expression in said cells;

(vi) calculating a cutoff value based on an average number of $MO2^+$ cells or average level of MO2 expression in samples obtained from healthy individuals; and

(vii) comparing the number of $MO2^+$ cells and/or level of expression of MO2 in said cells measured in (v) above to said cutoff value, a measured number of $MO2^+$ cells and/or level of expression of MO2 in said cells higher than the cutoff value, indicating a high probability of the existence of an infection in said tested individual.

The term *"healthy individual"* relates to an individual which does not have a bacterial or viral infection or any other detectable disorder or disease.

The level of expression of $MO2^+$ cells in healthy individuals will typically be determined by obtaining a blood sample from a large number of healthy individuals, determining the number of MO2+ cells and/or level of

MO2 expression in each sample as described above, calculating an arythmetic average of the values determined in each sample and adding a value of one standard deviation to said calculated average to obtain a *"cutoff value"* (one based on the number of $MO2^+$ cells and another on the level of MO2 expression). The value of the measured number of MO2+ cells and/or level of MO2 expression in cells of the tested individual are then compared to the corresponding Cutoff value. Where the measured value is higher than the cutoff value, there is a high probability of the existence of an infection in the tested individual.

The cutoff value may be predetermined for a certain set of conditions such as the kind of assay used, the reagents used etc., and should correlate with the conditions used in the method. Thus, it will be clear to a person versed in the art that, under certain conditions it may be necessary to add, for example, more than one standard deviation value to the average value to obtain the correct cutoff value.

In accordance with a preferred embodiment of the above aspects of the invention, following, or simultaneously with the incubation of the fixed and permeabilized mononuclear cells with anti MO2 MAbs, the cells are also incubated with MAbs capable of binding to additional T-cell antigens (such as CD3, CD4 and CD8) under conditions enabling binding of these MAbs to said antigens. Following double staining of the cells, the number of $MO2^+$ cells which also bind the additional MAbs (hereinafter *"double positive $MO2^+$ cells)* in said sample as well as the level of the MO2 expression in such cells are determined; a higher number of double positive cells and/or a higher level of MO2 expression in such cells compared to the number of double positive (which bind the same additional Mab) $MO2^+$ cells and/or the level of MO2 in such cells in control non-infected individuals, indicating a high probability of the existence of an infection in said tested individual.

As explained above, the level of MO2 expression in healthy individuals will typically be expressed as a cutoff value calculated as explained above and the measured value of the double positive cells will be compared to the cutoff value.

Where necessary, the significance of the difference between the measured value and the cutoff value may be analysed by any of the known Statistical tests such as, for example, Students' t-Test.

In accordance with the invention, the term *"infection"* relates to any infection of a bacterial origin such as, for example, pneumonia or urinary track infection (UTI), which often results in sepsis. In addition, an infection may be of a viral origin such as, for example, an HIV infection.

The separation of mononuclear cells from peripheral blood obtained from a tested individual may be carried out by any of the methods known in the art such as for example separation on a Ficoll-Hypaque gradient.

The detection of the MO2 antigen in accordance with the invention may be carried out by using any antibody capable of binding to the MO2 antigen such as, for example, the MO2-RD1 antibody used in the examples below. The antibody used may be directly labeled with a detectable marker such as radioactive or fluorescent marker. Alternatively, the anti MO2 antibody may be detected by using a secondary antibody comprising a detectable label.

In accordance with the invention, the anti MO2 antibody used for detecting the MO2 antigen may be a whole antibody or alternatively, fragments of the anti MO2 Mab having similar binding properties. Such Mab fragments may be for example, an Fab fragment or an $F(ab')_2$ fragment obtained by cleavage with appropriate enzymes as known in the art. Fragments which may be useful in accordance with the invention may be screened by competitive binding assays with the respective whole anti MO2 MAb and those which are capable of competing with such binding, i.e. bind

to the MO2 antigen may be used. The MAbs in accordance with the invention may belong to any of the various immunoglobulin classes.

The binding of the anti MO2 MAb may be detected in accordance with the invention by any of the methods known in the art such as FACS, RIA, ELISA, immunoblotting, etc. For detection of intracellular expression of the MO2 antigen in the cells in accordance with the invention, the tested cells are fixed and permeabilized prior to their staining with the relevant MAb by methods known in the art.

The detection of the additional T-cell antigen in the tested cells may be carried out by using any of the anti-T-cell specific antibodies known in the art (such as anti-CD3 for detecting T-cells, anti-CD4 for detection of helper T-cells or anti-CD8 for detection of suppressor T-cells) as well as any fragments thereof or labeled antibodies that essentially maintain the binding characteristics of the whole non labeled antibody. The detection of the binding of such antibodies to the cells may be carried out by any of the methods known in the art such as those mentioned above.

In addition, in some cases cells were found in accordance with the invention to be in a *"reactive"* state. The term *"reactive"* relates to any kind of reaction of the cells which results in intracellular changes as compared to the cells in their resting form. The reactive state may be of various kinds including, for example, an apoptotic reaction of the cells. The internal expression of the MO2 antigen in the cells may be a result of an internal signal in the cells or may result from the cells' response to an external signal of some kind (e.g. soluble MO2 antigen) and this signal may, in some cases, be taken up by the cells from their environment. In accordance with the invention, it has been shown that the intracellular expression of the MO2 antigen in $MO2^+$ cells may be detected even in relatively slightly reactive cells, (such as, for example, was shown in gamma/delta positive $MO2^+$ cells following their growth in culture (see example below).

In accordance with an additional aspect of the invention, it has been shown that the level of $MO2^+$ cells may be a useful marker for monitoring the efficacy of a certain treatment administered to an in individual suffering from an infection. As mentioned above, infected individuals show a higher level of $MO2^+$ cells before receiving treatment as compared to the level of $MO2^+$ cells in healthy individuals. In accordance with the invention, it was shown that, following treatment, the effect of the treatment may be monitored in the infected individuals on the basis of the level of their $MO2^+$ cells following treatment. Routinely, the effect of treatment of a viral infection is monitored by measuring the viral load in the treated individual. In accordance with the invention, it has been shown that even when the viral load of treated infected individuals decreases in all the treated individuals, in some, the level of the $MO2^+$ cells remains significantly higher than the level of the cells in healthy individuals, while in other individuals following treatment, the level of MO2 cells is reduced to the level of these cells in healthy individuals. Thus, the level of $MO2^+$ cells in treated infected individuals may be used as a basis for determining, on a cellular level, the effect of the treatment on the treated individual.

Thus the present invention further provides a method for monitoring the efficacy of a treatment in an infected individual comprising:

(i) obtaining a peripheral blood sample from said individual prior to administration of said treatment;

(ii) separation of mononuclear cells from said peripheral blood;

(iii) fixation and permeabilization of said mononuclear cells;

(iv) incubation of said fixed and permeabilized mononuclear cells with MAbs which bind to the MO2 antigen under conditions enabling binding of the MAbs to said antigen;

(v) detecting binding of said antibodies in said cells, and determining the number of cells in said sample expressing the

MO2 antibody internally and/or the intracellular level of MO2 expression in said cells;

(vi) administering said treatment to the individual;

(vii) at various periods of time following said treatment obtaining a peripheral blood sample from said treated individual;

(viii) determining the number of cells in said samples expressing the MO2 antigen internally and/or the intercellular level of MO2 expression in said cells as in step (ii) – (v) above;

(ix) comparing the number of said cells and/or said level of expression of MO2 in the cells in the sample obtained in (i) to the number of said cells and/or the level of MO2 expression in samples obtained from said individual following treatment, a significantly different number of $MO2^+$ cells or a significantly different level of expression of MO2 in cells present in samples obtained from said treated individual as compared to the number of $MO2^+$ cells or the level of MO2 expression in cells in a sample obtained prior to said treatment indicating efficacy of the treatment.

The term "*significantly different*" relates to either the number of $MO2^+$ cells or to the level of expression of MO2 in the cells which may be higher or lower than the number of $MO2^+$ cells or the level of expression of MO2 in healthy individuals. The significance of the difference will be determined by known Statistic tests. The measured value obtained from the tested individual may be compared to a cutoff value calculated from samples of healthy individuals as explained above.

By another of its aspects, the present invention provides a kit comprising antibodies which bind to the MO2 antigen together with reagents necessary for fixation, and permeabilization of the tested cells and, optionally, additional antibodies capable of binding to T-cell antigens and means for

detecting said binding of said MAbs to the tested cells. Optionally, the kit will also comprise cutoff values to which the measured level of $MO2^+$ cells and/or level of MO2 expression are compared.

EXAMPLES

The invention will now be illustrated further by the following examples with occasional reference to the figures. The examples are intended for illustration purposes only and are not to be construed as limiting.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows the results of fluorescence analysis by FACS of mononuclear cells obtained as described below which were first fixed and permeabilized as explained below, and then stained using one or more of the following MAbs:

Fig. 1A MY4-FITC

Fig. 1B MO2-RD1

Fig. 1C IgG2-FITC (control for MY4-FITC); and

Fig. 1D IgM-RD1 (control for the MO2-RD1 MAb).

The figure shows the level of fluorescence on the stained cells as well as the size of the cell. The cells are considered to be positively stained wherein the level of fluorescence of the cells is above the fluorescence level of the same cells after their incubation with the control MAbs.

Fig. 2 shows FACS analysis of cells stained by the MAbs mentioned in Fig. 1 without fixation or permeabilization of the cells prior to staining, i.e. the membrane expression of the antigens.

Fig. 3 shows results of FACS analysis of lymphocytes stained with the control IgM-RD1 Mab or MO2-RD1 MAb either after fixation only (Figs. 3A and 3C) or after fixation and permeabilization (Figs. 3B and 3D).

Fig. 4A shows results of FACS analysis of lymphocytes following double staining of the cells with the MO2-RD1 MAb and anti CD3-FITC MAb;

Fig. 4B shows FACS analysis of the lymphocytes stained with the control MAb IgM-RD1 and the anti CD3-FITC MAb.

Fig. 5 shows the results of FACS analysis of double staining of lymphocytes with the MO2-RD1 MAb and anti CD8-FITC MAb (Fig. 5A). IgM-RD1 was used as control MAb (Fig. 5B).

Fig. 6 shows results of FACS analysis of double staining of the lymphocytes with the MO2-RD1 MAb and an anti CD4-FITC MAb (Fig. 6A) the IgM-RD1 antibody was used as control MAb (Fig. 6B).

Fig. 7 shows FACS analysis of double staining of lymphocytes with the MO2-RD1 MAb and anti gamma/delta TCR-FITC MAb (Fig. 7A). The IgM-RD1 antibody was used as control (Fig. 7B).

Fig. 8 shows FACS analysis of double staining of lymphocytes with the MO2-RD1 MAb and an anti CD22-FITC MAb (Fig. 8A). The IgM-RD1 MAb was used as a control Mab (Fig. 8B).

Fig. 9 shows the results of FACS analysis of lymphocytes stained with the anti CD16-FITC MAb and MO2-RD1 MAb (Fig. 9A). The IgM-RD1 MAb was used as a control MAb (Fig. 9B).

Fig. 10 is a schematic representation showing the percent of the lymphocytes expressing the MO2 antigen intracellularly in mononuclear blood cells obtained from individuals suffering from various kinds of infections (black bars in the fig.) as compared to the presence of $MO2^+$ cells obtained from healthy control individuals (grey bars in the fig.). The expression of the MO2 antigen was detected using the MO2-RD1 MAb on fixed and permeabilized cells as described below. The results show the total $MO2^+$ lymphocyte population, the $MO2^+CD3^+$ lymphocyte population and the $MO2^+$ $CD3^-$ lymphocyte population in both above groups of individuals.

Fig. 11 is a schematic representation showing the percent (and mean percent + S.D.) of $MO2^-$ $CD8^+$ cells in four individuals suffering from various kinds of infections as compared to the percent (and mean percent + S.D.) of $MO2^+$ $CD4^+$ cells in the same individuals.

Fig. 12 is a schematic representation showing the percent of $MO2^+$ $CD3^+$ cells in ten individuals suffering from various kinds of infections as compared to the percent of these cells in healthy individuals.

MATERIALS AND METHODS

Fixation and permeabilization of lymphocytes:

Peripheral blood is obtained from individuals to be tested and mononuclear cells are separated on a ficoll-hypaque gradient. The cells are then washed in phosphate buffered saline (PBS) and then fixed in 4% paraformaldehyde in PBS for 30 mins. at 4°C. The cells are then washed again in PBS and permeabilized in PBS supplemented with 0.1% saponin and 1% bovine serum albumin for 30 mins. at room temperature.

Staining:

The cells prepared as described above, are then divided into staining tubes and processed in the saponin buffer for 1 or 2-color fluorescent staining. The cells are incubated with the tested monoclonal antibody or with the control monoclonal antibody for 30 mins. at room temperature. Staining can be done either by direct staining, i.e. by incubating the cells with a labeled Ab, or indirectly, i.e. by incubating the cells with first non-labeled Ab and then with a secondary labeled Ab. Following incubation, the cells are washed once in saponin buffer and twice in PBS. The level of staining is then determined using the FACS as described below.

Monoclonal antibodies:

The MAbs used to determine the expression of various antigens in the tested cells are the following:

The anti-CD14 MAbs anti-MY4 and anti-MO2 as well as the Anti-CD3, Anti-CD4, Anti-CD8, and Anti-CD16 were all obtained from Coulter Immunology. The anti-TCR, alpha beta, and anti-TCR gamma delta MAbs were obtained from Immunotech.

Fluorescence-activated cell Sorter-FACS

The analysis of the intracellular or membrane expression of the MO2 antigen is performed with the use of the FACS (model FACSCAN manufactured by Becton Dickinson) or FACS CALIBUR.

Cells of the lymphocyte or monocyte lineage can be detected and characterized according to their light-scattering properties. Their scatter positions, determined by their different forward and side laser- light scatters, enable to analyze both populations independently, by applying a "*gate*" or window on the cells of interest. In addition, the instrument is capable of measuring the fluorescence of the cells, either the green fluorescence, emitted by FITC, or the red fluorescence, emitted by RD1 – two different dyes conjugated to the MAbs. On the basis of the fluorescence of the cells, data can be obtained on the number of fluorescent cells, above background, in the population of cells under analysis, and also on the level of intensity of the fluorescence, a measurement directly correlated with the amount per cell of the antigen detected by the conjugated MAb.

RESULTS:

Example 1: <u>Intracellular expression of MO2 in lymphocytes</u>

As seen in Fig. 1, the novel population of lymphocytes which express the MO2 antigen internally was detected using the MO2-RD1 antibody on

fixed and permeabilized mononuclear cells which were obtained as described above. No expression of an antigen recognized by the MY4-FITC antibody was detected on the same cells. As seen in Fig. 2, no expression of the MO2 antigen or the MY4 antigen was detected in the same cells which were not fixed and permeabilized, i.e. the MO2 antigen which was expressed internally on the cells was not detected on their surface.

As seen in Figs. 1 and 2, the MO2 antigen was expressed to some extent on the cell surface of monocytes.

Example 2: **Detection of intracellular expression of MO2 on lymphocytes by fixation and permeabilization.**

Mononuclear cells obtained as explained above, were divided into the following two groups:

1. Cells that underwent fixation only as described above;
2. Cells that were fixed and also permeabilized as described above.

Both groups of cells were incubated with the MO2-RD1 MAb and the expression of MO2 was analzyed by FACS.

As seen in Fig. 3, the MO2 antigen was detected only in the above group 2 lymphocytes and no antigen was detected in cells of group 1. The above results show that the cells express the MO2 antigen internally only and that detection of the MO2 antigen in these cells by the Anti-MO2 MAbs requires permeabilization of the cells.

Example 3: **The $MO2^+$ cells comprise two subpopulations:**

3.1 Mononuclear cells obtained as explained above were double stained with an anti-MO2-RD1 antibody and an anti-CD3-FITC MAb and the level of fluorescence was measured by FACS as explained above. As seen in Fig. 4, the $MO2^+$ cells comprise

two subpopulations: those which do not express the CD3 antigen (non T cells, hereinafter $CD3^- MO2^+$ (Fig. 4A) and those cells expressing the CD3 antigen (T lymphocytes, hereinafter $CD3^+ MO2^+$ (Fig. 4A).

3.2 <u>Characterization of the $CD3^+ MO2^+$ T lymphocytes</u>:

3.2.1 Mononuclear cells were double stained with the anti MO2 MAb and an anti-CD8 MAb (a marker of cytotoxic and suppressor cells). As seen in Fig. 5, a large percent of the $CD8^+$ cells also express the MO2 antigen internally.

3.2.2 The mononuclear cells were double stained with the anti MO2 MAb and an anti-CD4 Mab (a marker for T helper cells). As seen in Fig. 6, only a very small pecent of the $CD4^+$ cells expressed the MO2 antigen internally.

3.2.3 The mononuclear cells were double stained with the anti MO2 Mab and with a MAb recognizing the gamma/delta T-cell receptor (about 2% of peripheral blood T cells). As seen in Fig. 7, most of the T cells expressing the gamma/delta type T cell receptor also expressed the MO2 antigen internally.

3.3 <u>Characterization of the non T $MO2^+ CD3^-$ cells:</u>

3.3.1 Mononuclear cells obtained as explained above were double stained with the anti MO2 MAb and an anti-CD22 MAb (which is a marker for mature B lymphocytes). As seen Fig. 8, none of the CD22+ cells (mature B lymphocytes) were positive for MO2. The above results show that none of the B cells express the MO2 antigen internally. These results also show that the

$CD3^-$ cells which express the MO_2 antigen internally (see 3.1 above) are non T- non B cells.

3.3.2 The cells were double stained with the MO2 Mab and with the anti-CD16 antibody (NK-like cells). As seen in Fig. 9, a large percent of the NK-like cells (CD16+) also express the MO2 antigen internally.

Example 4: **Expression of $MO2^+$ cells in individuals suffering from infection:**

4.1 The percent of the cells expressing the MO2 antigen internally ($MO2^+$) in individuals suffering from various infectious diseases of bacterial and viral origin were determined and compared to the percent of the $MO2^+$ cells in healthy individuals. As seen in Fig. 10, the percent of $MO2^+$ lymphocytes in individuals suffering from infections was substantially higher than the percent of these cells found number of the cells in healthy individuals. As may be seen, the most substantial rise is in the percent of $CD3^+$ $MO2^+$ cells.

4.2 As seen in Fig. 11, the level of $MO2^+$ $CD3^+$ cells was found to be higher in individuals suffering from various kinds of infections including bacterial (sepsis) and viral (asymptomatic and symptomatic HIV+ individuals). The mean percent of $MO2^+$ $CD3^+$ cells in healthy individuals was in the range of between about 0% to 15% while the mean percent of these cells in individuals suffering from infection was in the range of between about 20% to about 60%.

4.3 In order to characterize the $MO2^+$ T cells appearing in a higher level in individuals suffering from infections as compared to healthy individuals lymphocytes obtained from four individuals

suffering from various infections were also stained with the MO2-RD1 MAb and with anti-CD8 or with anti-CD4 antibodies. As seen in Fig. 12, in each of the four tested individuals, the level of the $MO2^+$ T cells expressing the CD8 antigen was substantially higher than the percent of $MO2^+$ T cells expressing the CD4 antigen in the same individual. Therefore, the majority of the $MO2^+$ T cells which are found at a higher level in individuals suffering from infections seem to be $CD8^+$ T cells while only the minority of these cells seem to be $CD4^+$ T cells.

Example 5: <u>Expression of intracellular MO2 in gamma/delta positive cells activated by growth in culture</u>

Mononuclear cells, obtained after Ficoll-Hypaque purification, were divided into two groups. Cells in group one were fixed, permeabilized and double stained for intracellular MO2 antigen and for the gamma-delta T-cell receptor antigen, as explained above. Cells in the second group were put in culture (RPMI medium, supplemented with 5% fetal-calf serum, glutamine and antibiotic mix), in a humidified incubator, for 18 hours, at 37 degrees and 5% CO_2. At the end of the incubation period, cells were washed, fixed, permeabilized and double stained for intracellular MO2 antigen and for the gamma-delta T-cell receptor antigen, as explained above.

Results, shown in Table 1, represent the net mean intracellular MO2 fluorescence of gamma-delta positive cells, before and after the incubation. The net mean fluorescence channel (obtained from the FACS analysis), correlates with the intracellular amount of the MO2 antigen. As shown, in the majority of the samples analyzed, we observed a significant increase in the amount of the intracellular MO2

antigen in the gamma-delta T-cells, following their stimulation by the 18 hours culture.

Table 1

Individual #	Mean Net Fluorescence Channel (Correlates with intracellular amount of MO2 antigen)	
	Before Culture	After Culture
1	2.3	4
2	2.5	11.7
3	1.5	12
4	2.7	9.7
5	3	17
6	17.8	20.6
7	0.9	6.6
8	1.5	12.8
9	3	13.3
10	2.7	13.9

Example 6: Monitoring the effect of treatment in HIV-positive individuals

6.1 The level of several populations of $MO2^+$ cells was determined in a number of healthy individuals as well as in asymptomatic untreated individuals which were determined as HIV-positive individuals on the basis of a positive serological test (based on positive binding of anti-HIV antibodies determined by ELISA and Western Blot assays). The level of three populations of $MO2^+$ cells $CD3^+$ $MO2^+$ cells, $CD4^+$ $MO2^+$ cells and $CD8^+$ $MO2^+$cells was determined by double staining of cells present in the samples obtained from the tested individuals with MO2 MAbs; anti CD4 MAbs or anti CD8 MAbs, respectively, as described above.

As seen in Table 2, the level of each of the above three $MO2^+$ populations was significantly higher in HIV-positive individuals as compared to the level of the same population of cells in healthy individuals.

Table 2

Healthy Individuals Age 21-60	% $CD3^+MO2^+$ 8.4% (SD 6.2) n = 23	% $CD4^+MO2^+$ 0.3% (SD 0.7) n = 11	% $CD8^+MO2^+$ 14.8% (SD 5.7) n = 11
HIV-Pos Individuals Age 25-54	31.3% (SD 11.3) n – 17	6.4% (SD 6.2) n = 17	51.6% (SD 11.5) n = 17

6.2 Monitoring the effect of anti-retroviral treatment of HIV-positive individuals on the basis of the level of $MO2^+$ cells:

Four HIV-positive individuals receiving anti-retroviral treatment comprised of a combination of three drugs (two nucleoside analogs and one protease inhibitor) were tested at various periods of times after the beginning of the treatment for their total viral loads as well as for the level of their $MO2^+$ cells. The level of viral load was determined by the NASBA-Nuclisense Nucleic Acid Sequence based amplification (Nuclisense Organon Teknika). The level of the four sub-populations of $MO2^+$ cells in the blood samples was detected by double staining of the cells as described above.

Results of the tests determining viral load in the treated individuals showed that the total viral load decreased in all four treated individuals following treatment (results not shown).

When testing for the level of $MO2^+$ cells, as seen in Table 3 below, in two HIV-positive treated individuals (designated as

AP and RA in the table) a significant decrease was seen in the level of $MO2^+$ cells following treatment of the individuals. The decrease was seen following a period of one month of the treatment. The level of the $MO2^+$ cells continued to decrease after three months and four months of the treatment. The level of the $MO2^+$ cells in these two treated individuals reached a level which was very similar (and in some cases even lower) than the level of the cells in healthy individuals.

However, as seen in the table, in the two remaining HIV-positive treated individuals (designated as GR and DO in the Table) the level of the $MO2^+$ cells did not decrease following treatment and in some cases the level of these cells even increased (see the level of $CD4^+$ $MO2^+$ cells in individual GR).

Table 3

	% $CD3^{+}MO2^{+}$				% $CD3^{-}MO2^{+}$				% $CD4^{+}MO2^{+}$				% $CD8^{+}MO2^{+}$			
HIV-Positive individual receiving anti-viral treatment	Before treatment	After treatment			Before treatment	After treatment			Before Treatment	After treatment			Before treatment	After treatment		
		1 mth	3 mth	4 mth		1 mth	3 mth	4 mth		1 mth	3 mth	4 mth		1 mth	3 mth	4 mth
A.P	45.7	20.7	13.3	9.4	75.4	14.2	25.3	23.9	25.4	0.4	0	0	62.8	26.2	18.5	15.4
R.A	51		13.5		53		25.2		5	2.6			61	33.5		
G.R	35.3	44.2	43.8		83.5	73.8	66.0		1.4	7.1			50.5	56.6	69.6	
D.O	23	34.7			70	41			10	10.9			46	52		

The above results show that the level of $MO2^+$ cells may be a preliminary marker for HIV infection. In addition, the level of these cells may be useful to monitor the effect of an antiviral treatment on a tested individual.